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The Development of the Endogenous Stages of Eimeria ninakohlyakimovae (Yakimoff and Rastegaieff, 1930) in Domestic Sheep

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THE DEVELOPMENT OF THE ENDOGENOUS STAGES OF *EIMERIA NINAKOHLYAKIMOVAE* (YAKIMOFF AND RASTEGAIEFF, 1930) IN DOMESTIC SHEEP

by

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ABSTRACT

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Eimeria ninakohlyakimovae (Yakimoff and

Rastegaieff, 1930) in Domestic Sheep

by

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Utah State University, 1970

Major Professor: Dr. Datus M. Hammond
Department: Zoology

Forty-five mixed breed lambs, 1 to 4 months of age, were used to study the patent period and endogenous stages in the life cycle of *Eimeria ninakohlyakimovae*. The lambs were inoculated with oocysts of this species and killed at daily intervals from 1 through 14 days after inoculation. From all lambs of the study sections of intestinal tissue were prepared by routine methods for histological examination.

E. ninakohlyakimovae was observed to have 2 generations of schizonts in its endogenous development. Mature, first-generation schizonts had an average diameter of about 290 µ and had many thousands of merozoites, averaging 11.9 by 2.1 µ. Macroscopically, these schizonts appeared as small white bodies in the mucosa of the ileum, with the largest numbers occurring 5 to 15 feet anterior to the ileocecal valve. Young, first-generation trophozoites, having a crescent body in the parasitophorous vacuole and

at least 1 refractile body, were observed as early as 3 days post-inoculation. These underwent development in cells of the lamina propria which were adjacent to the base of the crypts of Lieberkuhn. As the schizonts grew, the adjacent wall of the crypt became indented. The cytoplasm of the host cell harboring the parasite increased in volume, and its nucleus and nucleolus became enlarged. The host cell then became surrounded by an envelope of flattened cells. Within the developing schizonts, the nuclei multiplied, and at about 7 days post-inoculation they became arranged in a single layer at the periphery. By a series of infoldings of this layer, compartments were formed within the schizont. The parasitophorous vacuole appeared to form inpocketings in association with the infoldings. The compartments later gave rise to spheroidal bodies (blastophores) having a single peripheral layer of nuclei. From the blastophores outgrowths occurred; these formed the first-generation merozoites. Mature first-generation schizonts appeared 9 to 10 days post-inoculation. Many mature schizonts became surrounded by leucocytic cells, which later invaded and destroyed the schizonts. Phagocytosis of the merozoites by macrophages in invaded schizonts was observed.

Mature, second-generation schizonts developed in epithelial cells lining the crypts in the large intestine 10 to 11 days after inoculation. Development required about 1 to 2 days. When mature, these schizonts averaged about 12 μ in diameter and had an average of 24 merozoites.

These merozoites averaged 5.5 μ by 1.4 μ. Many appeared to have a crescent-shaped distribution of chromatin in their nuclei. The merozoites developed in a manner similar to that in individual blastophores of first-generation schizonts. A crescent body was present in second-generation schizonts.

At 11 through 14 days post-inoculation, sexual stages were observed in the epithelial cells lining the crypts of Lieberkuhn in the large intestine. Thirty mature microgametocytes averaged 15.0 by 11.6 μ. In mature microgametocytes, the microgametes were arranged peripherally about a central residual mass. Thirty mature macrogametocytes averaged 16.1 by 12.3 μ. Within the nucleus of young macrogametocytes, a basophilic body (satellite body) was observed adjacent to the nucleolus. At maturity, the cytoplasm of the macrogametocytes contained numerous eosinophilic and basophilic granules. A crescent body was present in the parasitophorous vacuole of both macrogametocytes and microgametocytes. Thirty unsporulated oocysts, in situ, averaged 17.6 by 13.3 μ. The prepatent period in 4 lambs was 11 days and the patent period in 2 lambs was 7 days.

The pattern of endogenous development of E. ninakohlyakimovae closely resembles that of E. bovis.

(66 pages)

INTRODUCTION

The coccidian parasite Eimeria ninakohlyakimovae Yakimoff and Rastegaieff, 1930, is highly pathogenic in sheep and goats, its effects being most noticeable in feed lot lambs. Balozet (1932) and Lotze (1954) investigated the life cycle of E. ninakohlyakimovae; their accounts do not agree. In parasite-free sheep, Lotze (1954) found apparent single-generation schizonts measuring about 300 μ and having numerous merozoites. Similarily, large schizonts were found to be associated with this species in lambs by Hammond, Kuta and Miner (1967). In goats, Balozet (1932) found schizonts measuring 15 to 35 μ and containing relatively few merozoites. Sayin (1964) also found small schizonts (15 to 40 μ) for this species in goats. These investigations also provided general information regarding the occurrence of the sexual stages. However, the exact location of these and of the schizont stages were not determined. It has been suggested that the smaller schizonts observed in goats may be of a different species or of a second generation after that of the large schizonts (Levine, 1961). More detailed work on this life cycle was needed to clarify these and other aspects.

For a more complete understanding of this parasite, detailed information as to the cytology of its large

schizont and other endogenous stages was needed. Hammond, Ernst and Miner (1966) presented detailed information on merozoite formation, host cell reaction, and the invasion by leucocytes of the first-generation schizont of Eimeria bovis. Since the large E. ninakohlyakimovae schizont is similar in size to that of E. bovis and also has numerous merozoites, a comparative study of the two appeared desirable to provide a greater understanding of the developmental processes involved.

The first objective of the present study was to obtain information concerning the development of the schizonts and merozoites, reaction of the host cell, and invasion of large E. ninakohlyakimovae schizonts by leucocytic cells and to compare this information with the data known about these processes in E. bovis and other species. The second objective was to obtain more detailed information about the sexual stages in the life cycle of E. ninakohyakimovae and to compare this information with that known about E. bovis and other species.

REVIEW OF LITERATURE

The coccidian parasite Eimeria ninakohlyakimovae Yakimoff and Rastegaieff, 1930, has been reported from several species of ruminants including the Rocky Mountain Bighorn sheep (Ovis canadensis), mouflons (Ovis musimon and Ovis orientalis), the Barbary sheep (Ammotragus lervia), the Siberian ibex (Capra ibex sibirica), and the Persian gazelle (Gazella subgutturosa). It has been reported most commonly from the various breeds of domestic sheep (Ovis aries) and domestic goats (Capra hircus) (Pellérdy, 1965). Experimental investigations regarding the endogenous stages of E. ninakohlyakimovae are few and have been conducted only on domestic sheep and goats.

The first of these experimental studies was that of Balozet (1932). In his study, Balozet observed the presence of schizonts and sexual stages in the epithelial cells of the small intestine of a young goat which had been inoculated with oocysts of E. ninakohlyakimovae. This goat was killed for study at about 40 days post-inoculation. The schizonts measured 15 to 35 μ in diameter and were reported to have 40 to 200 merozoites. The merozoites measured 1.5 to 2.0 μ in diameter. The diameter of the microgametocytes was found to be 45 to 50 μ, but the macrogametocytes were not measured. In the tissue, schizonts were found to occur in association with the

gametocytes and oocysts. In his description of the sexual stages, Balozet reported a pattern of development for microgametocytes and macrogametocytes similar to that reported for other species of coccidia.

A second study involving goats was that of Sayin (1964), who examined tissues taken from a young goat that was passing only oocysts of E. ninakohlyakimovae. Schizonts were found to occur in association with the sexual stages in the epithelial cells of the small intestine, cecum, and large intestine. The schizonts measured 22 to 43 μ in diameter and had an undetermined number of merozoites which measured 11 by 1 μ. The microgametocytes had a mean diameter of 22.5 by 16.5 μ, and macrogametes, a mean diameter of 13.5 by 10.0 μ.

Information about the endogenous stages of E. ninakohlyakimovae in sheep was provided by Lotze (1954) in his study regarding the pathogenicity of the parasite. In this study, tissues of 12 3-month-old lambs which had been inoculated with 500,000 to 5,000,000 oocysts of E. ninakohlyakimovae were examined microscopically. All lambs had been raised parasite-free before inoculation. They were killed for study on alternate days from 1 day through 19 days post-inoculation. Schizonts were reported to undergo development in the lower part of the small intestine, and the host cells were considered to be endothelial cells at the base of the crypts of Lieberkuhn. Young schizonts were observed as early as 5 days post-inoculation and mature schizonts as late as 13 to 15 days post-

inoculation. Sexual stages of the parasite were reported to occur in the epithelial cells of the ileum, cecum, and upper part of the large intestine.

Further information concerning the endogenous stages of E. ninakohlyakimovae in sheep was reported by Hammond et al. (1967), in a drug study of coccidiosis. In this study, lambs were inoculated with 50,000 to 100,000 oocysts of E. ninakohlyakimovae (97%) and E. intricata and E. parva (3%). Large schizonts, having an average size of 203.9 by 150.8 μ, were observed in the lamina propria of the ileum. Several of these schizonts had become invaded by leucocytic cells. The sexual stages occurred characteristically in the epithelial cells of the lower small intestine and in the large intestine. These large schizonts and sexual stages were assumed to be those of E. ninakohlyakimovae. Small schizonts similar to those of the second generation of the present study were not reported either by Lotze (1954) or by Hammond et al. (1967).

Several studies of the endogenous stages of E. bovis in cattle are relevant to the comparative aspects of the present paper. These studies include that of Hammond et al. (1946), who investigated the endogenous stages in the life cycle of E. bovis, and a study in which the occurrence of a second asexual generation in that life cycle was described (Hammond, Anderson and Miner, 1963), a cytological study of the first-generation merozoites of E. bovis (Hammond, Ernst and Goldman, 1965), and the development of the first-generation schizonts of E. bovis (Hammond, Ernst

and Miner, 1966).

MATERIAL AND METHODS

Forty-five lambs, 1 to 4 months of age, were used in the present study. All lambs were mixtures of Columbian, Rambouillet, Hampshire, and Suffolk breeds. A total of 28 lambs 1 week of age or less were obtained during the spring of 1967, --68, and --69 and each was placed with its ewe in an individual pen. These lambs were kept with their ewes throughout the experiment; they were inoculated when they were 3 to 4 weeks old. The ewes were not treated for coccidiosis at any time during the experiment. Also, a total of 17 lambs 3 to 4 months old were obtained during the fall of 1967 and --68 and were maintained individually in pens without their ewes.

Each pen was scrubbed down with detergent and provided with fresh straw before use. The straw was replaced twice weekly. All pens were out of doors and were partially covered with a sloping roof. Each pen had a dirt or a crushed rock floor which measured about 4 feet by 8 feet. All sheep had daily access to dry alfalfa, mixed grain, and water.

Two lambs were each inoculated with 5,000 to 10,000 oocysts of Eimeria ninakohlyakimovae in order to determine the prepatent and patent periods of infection and to obtain additional inoculum. These lambs were later reinoculated with higher dosages of oocysts and killed for

tissue stages of the parasite. An inoculum consisting only of E. ninakohlyakimovae oocysts was used in the present study. It was obtained from lambs inoculated with a supply of oocysts made available from an earlier study by Hammond et al. (1967).

In order to obtain sufficient numbers of trophozoites of first-generation schizonts for study, an intestinal fistula was prepared in each of 2 4-month-old lambs according to the methods of Hammond et al. (1946) and Chobotar, Hammond and Miner (1969). Each fistula in the present study was prepared from a 12-inch section of the ileum surgically isolated from the intestine at a point 10 feet anterior to the ileocecal valve. Five days after surgery, each fistula was inoculated with sporozoites excysted from 5,000,000 oocysts of E. ninakohlyakimovae. The oocysts were cleaned, pretreated, and excysted according to the methods of Hibbert and Hammond (1968). The excysted sporozoites were introduced into the fistula by pipette. One fistula lamb was killed at 2½ days post-inoculation; the second at 3 days post-inoculation. Sections of tissue from each fistula were prepared for histological examination by the methods described below.

In order to obtain sufficient numbers of trophozoites of second-generation schizonts for study, cecal biopsies were performed according to the methods of Hammond et al. (1963). In the present study, the cecum of each of 2 4-month-old lambs was ligated and then inoculated with

50,000,000 first-generation merozoites. Mature first-generation schizonts dissected out of the intestinal tissue of 2 lambs inoculated 10 days earlier with oocysts of E. ninakohlyakimovae provided the merozoites for inoculation. The inoculum was prepared according to the methods of Hammond et al. (1963). Merozoites were introduced into the cecum by syringe. The interval between the killing of the lambs used to provide merozoites and the introduction of these merozoites into the cecum of the biopsy lambs was 1½ to 2 hours. Cecal biopsies were performed at 24 and 48 hours after the introduction of merozoites into the cecum of one lamb and 36 hours after the introduction of merozoites into the cecum of the other. Biopsies performed before the inoculation of merozoites in both lambs were used as controls. Sections of tissue from each biopsy were prepared for histological examination as described below.

The remaining endogenous stages of the parasite were obtained from 41 lambs inoculated per os with 50,000 to 1,000,000 oocysts of E. ninakohlyakimovae. A nippled bottle was used to administer the inoculum. Fecal samples were collected intermittently from uninoculated lambs and daily from inoculated lambs. The samples were examined for coccidian oocysts by a modified McMaster technique (Whitlock, 1948). Lambs were killed at daily intervals ranging from 1 day after inoculation through 14 days. Sections of tissue for histological examination were

prepared routinely from the thyroid gland, adrenal gland, liver, kidney, mesenteric lymph nodes, abomasum, cecum, upper colon, middle colon, lower colon, and from the ileum at 5-foot intervals.

All tissues prepared for histological examination were fixed in formalin or Zenker's fluid, sectioned in paraffin, and stained with haematoxylin and eosin and with iron haematoxylin. Tissues were also prepared according to the methods of Feulgen (Barka and Anderson, 1963) and of Himes and Moriber (1956).

For the study of merozoites, living first-generation schizonts were obtained from lambs harboring 10-, 11-, or 12-day experimental infections. Individual schizonts were freed from the lamina propria of the intestine by dissection. Because the first-generation schizonts of E. ninakohlyakimovae occur deep in the lamina propria, attempts to free them from the tissue by scraping the mucosal surface failed.

Fresh and fixed first-generation merozoites were prepared from the schizonts according to the methods of Hammond et al. (1965). Living merozoites were examined by means of bright field, phase-contrast, and fluorescence microscopy. Merozoite smears for permanent mounts were fixed in Zenker's fixative and stained with iron haematoxylin and with haematoxylin and eosin. Feulgen and periodic acid-Schiff (PAS) preparations were made as described by Barka and Anderson (1963). PAS control smears were pretreated with 1% diastase in a 0.85% saline solution at 37 C

for one hour. Living merozoites were measured with bright field microscopy at a magnification of 1000 x.

Drawings were made with the aid of a camera lucida and photographs with the aid of a Zeiss photomicroscope. All measurements are in microns unless otherwise indicated; measurements given in parentheses refer to range. Measurements of prepared materials were made using tissues fixed in Zenker's fixative and stained with iron haematoxylin, haematoxylin and eosin, or with the method of Feulgen. All measurements were made with the aid of an ocular micrometer.

RESULTS

Duration of Experimental Infection

In each of 2 lambs used for inoculum, the passage of unsporulated oocysts in the feces began 12 days after inoculation, and each lamb had a continuous oocyst discharge from 12 to 18 days after inoculation. No oocysts were observed in the feces on, or after, day 19. Thus, the prepatent period was 11 days and the patent period 12 to 18 days. The peak number of oocysts discharged occurred at a mean of 13.5 (13 to 14) days after inoculation. Two other lambs in the study also had a prepatent period of 11 days. The two lambs used for inoculum became reinfected when reinoculated 1 month later for tissue sections.

Development of First-Generation Schizonts

First-generation schizonts underwent development in the reticular connective tissue cells of the lamina propria in the small intestine. Parasites were seen only in those reticular cells which were a part of the supporting envelope of connective tissue cells immediately surrounding and adjacent to the base of the crypts of Lieberkuhn (Figures 1, 2, 10 and 28). The host cell harboring the developing schizont characteristically indented the adjacent epithelial layer of the crypt so that it bulged into

the crypt lumen. The largest numbers of first-generation schizonts were observed in sections of tissue from the 5-, 10-, and 15-ft levels of the ileum anterior to the ileocecal valve, with smaller numbers occurring in some sections from the 20- and 25-ft levels. Living, mature schizonts were visible macroscopically as small bodies beneath the mucosal surface of the intestine. Fifty living, mature schizonts measured 290 (241.0 to 330.5) by 232.0 (188.0 to 285.0) and had many thousands of merozoites.

Trophozoites and early schizonts

The earliest observed endogenous stages were trophozoites in intestinal fistulas from one lamb killed 2½ days after inoculation and one killed 3 days after inoculation (Figures 4 and 27). Very few trophozoites were observed in the 2½-day fistula; in the 3-day fistula, trophozoites were more numerous. In tissues from lambs inoculated per os and killed 3 days after inoculation, no trophozoites were seen, but a few multinucleate schizonts were observed. However, multinucleate schizonts were seen more frequently in the fistula of the lamb killed 3 days after inoculation (Figure 28). No stages were observed in lambs inoculated per os and killed earlier than 3 days after inoculation. Five trophozoites averaged 8.5 (7.5 to 8.8) by 6.0 (5.0 to 6.3). Within each trophozoite, a single refractile body was observed as well as a nucleus, which

contained a nucleolus. No trophozoites or young schizonts were seen which had more than 1 refractile body. A crescent body was present within the parasitophorous vacuole surrounding the trophozoite. In trophozoites stained with iron haematoxylin, the nucleoplasm stained more intensely and more homogeneously than did the cytoplasm, which appeared light gray. The nucleolus stained more intensely than did the nucleoplasm and appeared in sharp contrast to it. Surrounding the nucleus, a clear, non staining area was usually present. With haematoxylin and eosin, the nuclei of trophozoites appeared similar to those stained with iron haematoxylin; as with iron haematoxylin, the nucleoli stained more intensely than did the nucleoplasm. Also, with haematoxylin and eosin, the pink staining cytoplasm of trophozoites was more granular in appearance than it was with iron haematoxylin. In the nucleoplasm of trophozoites stained with the Feulgen method, numerous Feulgen-positive granules were present surrounding the nucleolus, which stained Feulgen-negative.

Refractile bodies of trophozoites stained intensely with iron haematoxylin and eosinophilic with haematoxylin and eosin. With both of these stains, refractile bodies stained more intensely at the margin than at the center. With the Feulgen method, refractile bodies appeared Feulgen-negative.

Crescent bodies appeared similar to refractile bodies in staining characteristics. The crescent bodies were

Feulgen-negative, and with both haematoxylin and eosin and with haematoxylin the greatest intensity of staining occurred at the margins of these structures.

The cytoplasm of the host cell appeared to be hypertrophied, as did the host cell nucleus. The nucleolus of the host cell was markedly enlarged, so that it was very prominent. Most of the chromatin of the host cell was thinly distributed at the inner surface of the nuclear membrane, but some small chromatin granules were scattered within the nucleoplasm. In nuclei of non-infected cells, the peripheral layer of chromatin appeared thicker, and the granules of chromatin in the nucleoplasm larger.

In tissues from lambs inoculated per os and killed 4 days after inoculation, numerous young schizonts, having as few as 3 or 4 and as many as 8 or 9 nuclei were observed (Figure 1). The characteristic indentation of the crypt epithelium adjacent to the developing parasite was more conspicuous than in the trophozoite stage. Both the crescent body and the single refractile body were observed in the young schizonts (Figure 1). The multinucleate schizonts seen in the 3-day fistula (Figure 28) were at a similar stage of development. The nuclei of these young schizonts showed staining reactions similar to those observed for nuclei of trophozoites. However, the nuclei of the young schizonts appeared somewhat smaller than those of trophozoites. In tissues of this stage stained with the Feulgen method, the nucleoplasm had numerous Feulgen-

positive granules surrounding a nucleolus which stained Feulgen-negative. Refractile bodies and crescent bodies of these and later stages stained as in trophozoites.

At this time in the development of the parasite, the relationship of the host cell to the cells of the supporting reticular connective tissue sheath which surrounded the crypt became apparent (Figures 1 and 28). The adjacent cells of the reticular sheath were attached directly to the host cell, apparently anchoring it in a relatively fixed position. Nuclei of the reticular sheath were seen to flank the host cell at its juncture with the reticular cells (Figures 1 and 2).

In tissue from lambs killed 5 days after inoculation, schizonts had numerous nuclei randomly distributed within the cytoplasm (Figures 2, 10 and 11). These nuclei appeared smaller than in previous stages and showed numerous Feulgen-positive granules in the nucleoplasm surrounding the nucleolus. At this and later stages, schizont nucleoli were difficult to see in iron haematoxylin and haematoxylin and eosin preparations, as the nucleoplasm stained more deeply. The cytoplasm of schizonts in this and later stages appeared more granular and heterogeneous than in previous stages. The cytoplasm also appeared quite vacuolated (Figures 2 and 11). A refractile body was present in the cytoplasm and a crescent body was present in the parasitophorous vacuole. The cytoplasm of the host cell by this time had formed into two distinct layers (Figures

? and 11). The inner layer was more granular in appearance and stained more densely than the outer. Also, a layer of flattened cells began to form around the free surface of the host cell (Figure 2). The relationship of the host cell to the reticular connective tissue sheath surrounding the crypts appeared to be similar to that of the previous stage (Figure 2).

Schizonts observed in lambs killed 6 days after inoculation also had a random distribution of nuclei (Figures 12 and 13). A refractile body and crescent body were present. The flattened envelope of cells completely surrounded the host cell in most specimens (Figure 13). By this time, the relationship of the host cell to the reticular cells surrounding the "parent" crypt usually became obscure. However, the schizont usually remained in relatively close proximity to its "parent" crypt (Figure 18).

Intermediate schizonts

In schizonts from lambs killed 7 days after inoculation, the nuclei appeared to be organized into layers which formed the walls of variously sized compartments within the schizont (Figures 3, 14 and 15). The formation of these compartments appeared to follow a definite pattern. The process of compartmentalization presumably began with the arrangement of the randomly distributed nuclei into a single layer of nuclei along the periphery of the schizont. Although such a stage was not observed

in the present study, Hammond, Ernst and Miner (1966) did observe such a stage in E. bovis. At various points along the periphery of a succeeding stage, infoldings of a nuclear layer were observed to have occurred (Figures 3, 14 and 15). These primary infoldings gave rise to secondary and tertiary infolding layers of nuclei, which randomly combined to produce the complex array of compartments having walls with a single layer of nuclei. The parasitophorous vacuole contained a fluid which stained more densely and homogeneously than did the cytoplasm of the parasite. This fluid was observed within the infoldings of nuclear layers, indicating that inpocketings of the parasitophorous vacuole were present in the interior of the schizont between adjacent layers of nuclei. Thus, the individual compartments came to lie within a network of spaces continuous with the parasitophorous vacuole. Each individual compartment consisted of an internal mass of schizont cytoplasm, which appeared granular and vacuolated, a peripheral layer of nuclei, and a limiting membrane derived from that of the schizont.

During this stage in the development of the parasite, the envelope of flattened cells completely surrounded the host cell (Figures 3 and 15). The host cell cytoplasm appeared thinly stretched around the schizont; similarly the host cell nucleus appeared flattened and compressed (Figure 7). Neither refractile bodies nor crescent bodies were observed at, or after this stage.

Further infolding resulted in formation of small spheres called blastophores which had a peripheral layer of nuclei (Figure 16). Schizonts in this stage were seen 8 days after inoculation. Some blastophores resembled elongate tubes rather than spheres, their shape presumably resulting from compression by adjacent blastophores. Each blastophore formed first-generation merozoites by a radial budding process. During formation of the merozoites, a nucleus of the peripheral layer was incorporated into each (Figure 17).

An envelope of flattened cells was present at this stage. The host cell cytoplasm, averaging 1 to 2 in thickness, appeared as a thin layer immediately beneath the envelope of flattened cells.

Mature schizonts

By 9 days after inoculation, formation of merozoites within the schizont appeared to be complete, or nearly so (Figures 18 and 19). For a brief period during their formation, merozoites appeared to retain a patterned arrangement within the schizont (Figure 18). However, the newly liberated merozoites then began to lose this arrangement (Figure 19). At 10 days after inoculation, schizonts with randomly distributed merozoites were seen (Figure 20). The envelope of flattened cells was still present at this stage, and the host cell appeared as in the previous stage.

Also, at this time nearly all of the mature schizonts were surrounded by an accumulation of leucocytic cells (Figure 21). The accumulation of leucocytic cells was first observed 10 days after inoculation and apparently occurred only when the schizonts reached maturity. The leucocytes then began to invade the schizont in increasing numbers (Figures 21, 22 and 23). Invaded schizonts were first seen in lambs killed 10 days after inoculation, and completely destroyed schizonts in lambs killed 12 days after inoculation. Thus, the minimum time necessary for complete destruction of invaded schizonts was about 2 days. At 10 to 13 days after inoculation almost no mature schizonts, having a random distribution of nuclei, were seen which were not surrounded by numerous leucocytic cells or which were not being invaded. About 90% of these schizonts showed some degree of invasion. Leucocytic cells most commonly associated with the invasion process were eosinophils, neutrophils, and macrophages. The envelope of flattened cells surrounding the schizont appeared to have broken down in the areas where leucocytes had penetrated the schizont. The breakdown of the envelope of flattened cells apparently coincided with the invasion of leucocytic cells.

The merozoites within invaded schizonts were evidently phagocytized by macrophages (Figure 5). Merozoites within macrophages appeared first to round up and then to lose their cytoplasmic outline. The nuclei of the phagocytized merozoites remained morphologically distinct longer than

did the cytoplasm; then they too disappeared. Thus, phagocytized merozoites were apparently destroyed by the macrophages. The invasion of schizonts by leucocytic cells was observed consistently 10, 11, 12, 13, and 14 days post-inoculation, and appeared to be a consistent phenomenon in all but 1 lamb having mature schizonts. Sites of recently destroyed schizonts had numerous macrophages and fibrocytic connective tissue cells (Figure 24). These sites, here termed schizont scars, were observed 12, 13, and 14 days post-inoculation.

First-Generation Merozoites

One hundred living, mature, first-generation merozoites from five different schizonts measured 11.9 (10.4 to 12.7) by 2.1 (1.7 to 2.3). The nucleus was located in the posterior one-third of the merozoite (Figures 25 and 26). Numerous PAS-positive granules occurred in the middle one-third of the merozoite, and a few such granules were observed posterior to the nucleus. In control sections, treated with diastase, these granules were PAS-negative. In acridine orange preparations about 5 to 7 ribonucleic acid (RNA)-positive granules were observed in the middle one-third of the merozoites, with one or two such granules appearing posterior to the nucleus. Also, in these preparations, deoxyribonucleic acid (DNA)-positive material was concentrated in 3 to 5 clumps at the margins of the nuclei. Flexing and gliding movements were observed in living merozoites. However, probing and pivoting movements were not

observed. About 3 to 5 wrinkles in the concave portion of the pellicle were observed in flexing merozoites (Figure 26).

Development of Second-Generation Schizonts

Mature first-generation merozoites gave rise to second-generation schizonts (Figure 6). Thirty mature, second-generation schizonts in tissue sections were 12.0 (9.6 to 15.0) by 9.0 (6.6 to 12.0) and had an average of 24 (22 to 30) merozoites. Thirty mature second-generation merozoites in tissue sections were 5.5 (4.8 to 6.6) by 1.4 (1.2 to 1.8) (Figure 6).

At 10 and 11 days after inoculation, second-generation schizonts were observed in epithelial cells lining the crypts of Lieberkuhn in the cecum, upper, middle, and lower colon (Figures 29, 30, 31, 32 and 33). Young second-generation schizonts were first seen 10 days after inoculation, and mature schizonts only on the eleventh day. Development appeared to take 1 to 2 days.

A few young and mature second-generation schizonts were observed in cells of the lamina propria. Five second-generation trophozoites averaged 5.5 (5.0 to 6.3) by 5.0 (3.8 to 6.3). Trophozoites of second-generation schizonts were observed to have a crescent body which lay within the parasitophorous vacuole (Figures 7 and 29). The nuclei of trophozoites stained basophilic with haematoxylin and eosin, but the staining appeared more intense at

the margins of the nuclei. A clear space surrounded the nucleus. The cytoplasm stained pink with haematoxylin and eosin. Trophozoites were seen only in the 24-hr cecal biopsy sections stained with haematoxylin and eosin. No stages were observed in 36- or 48-hr cecal biopsies, or in the controls. Binucleate stages were observed in association with the trophozoites (Figure 30). The nuclei of binucleate stages appeared similar to those of trophozoites.

In the intermediate stages of development of young second-generation schizonts, nuclei were randomly distributed. At a later stage, the nuclei became arranged at the periphery of the developing schizont (Figures 31 and 32). Over each peripherally located nucleus, an elevated area appeared at the surface of the schizont (Figure 32). Each elevated area represented the site of a newly forming merozoite, which grew radially into the parasitophorous vacuole, incorporated its respective nucleus and pinched off to form a new merozoite (Figure 33). A central or eccentric residual body was formed (Figures 6 and 33).

Mature second-generation schizonts had a crescent body, a residual body, and merozoites (Figures 6 and 33). Mature second-generation merozoites showed Feulgen-positive nuclear material, which appeared crescentic (Figure 6). This suggested a marginal distribution of chromatin material at one side of the nucleus. Small nucleoli were observed within the nuclei of second-generation merozoites (Figure 6). The host cell appeared as a thin layer of

cytoplasm around the developing schizont, and the host cell nucleus was characteristically indented in the area adjacent to the schizont. No appreciable increase in the quantity of the host cell cytoplasm was observed. The nuclei of many mature second-generation schizonts appeared pycnotic (Figure 33).

Development of Microgametocytes

Microgametocytes were observed in the epithelial cells lining the crypts of Lieberkuhn in the cecum, and in the upper, middle, and lower colon. Thirty mature microgametocytes in tissue sections averaged 15.0 (9.0 to 22.2) by 11.6 (7.8 to 15.0).

Microgametocytes were observed in the tissues from 11 to 14 days after inoculation (Figures 9 and 34 to 39). A crescent body was present in the parasitophorous vacuole (Figures 9 and 35). Nuclei of young microgametocytes stained basophilic with haematoxylin and eosin, and appeared to stain more intensely at the margins with both haematoxylin and eosin and with iron haematoxylin. Several Feulgen-positive granules were present in the nucleoplasm of microgametocytes stained with the Feulgen method. A thin layer of host cell cytoplasm surrounded mature microgametocytes; there appeared to be no appreciable increase in the amount of cytoplasm. The host cell nucleus was characteristically indented in the region adjacent to the parasite. During the process of microgametogenesis, the

nuclei of young microgametocytes underwent a series of divisions and became randomly distributed within the microgametocyte (Figures 34, 35 and 36). The nuclei subsequently assumed a peripheral distribution (Figure 37), and then elongated as microgamete formation began. A large residual body was present after completion of microgamete formation (Figure 38). Mature microgametes appeared to retain their peripheral arrangement in the microgametocyte (Figure 39). Mature microgametocytes were first seen 12 days after inoculation.

Development of Macrogametes

Macrogametes were observed to undergo development in the epithelial cells lining the crypts of Lieberkuhn of the cecum, upper, middle, and lower colon. Thirty mature macrogametes in tissue sections averaged 16.1 (13.2 to 18.0) by 12.3 (10.2 to 14.4). Thirty young oocysts in tissue sections averaged 17.6 (15.0 to 20.4) by 13.3 (11.4 to 15.0).

Macrogametes were observed in the tissue from 11 to 14 days after inoculation. In young macrogametes a prominent nucleolus and associated satellite body were observed. The nucleolus stained intensely with iron haematoxylin and basophilic with haematoxylin and eosin, but the satellite body stained less intensely with iron haematoxylin than did the nucleolus and was eosinophilic with haematoxylin and eosin. The satellite body was Feulgen-negative. Both the

nucleolus and the satellite body were present in a large clear zone of nucleoplasm. The cytoplasm of young macrogametes appeared granular.

The nucleus of mature macrogametes had a small but prominent basophilic nucleolus. The margin of the nucleoplasm stained basophilic, giving a ring-like appearance to the nucleus (Figure 41). A thin layer of host cell cytoplasm surrounded mature macrogametes as in the microgametocytes. Similarly, the nucleus of the host cell appeared indented in the region adjacent to the macrogamete. A crescent body was present in the parasitophorous vacuole (Figure 40). In the cytoplasm of mature macrogametes two kinds of granules, eosinophilic and basophilic (Figure 41), were observed. The basophilic granules, which appeared dark in haematoxylin and eosin preparations, became arranged at the periphery of the macrogametocyte and then coalesced to form one layer of the oocyst wall. Mature macrogametes were first observed 12 days after inoculation. In completely formed oocysts, granules could no longer be seen in the cytoplasm (Figure 42).

Figures 1-9. Drawings of endogenous stages of E. ninakohlyakimovae from sections of the intestine of lambs fixed with Zenker's fluid and stained with haematoxylin and eosin unless otherwise noted. Figures 1-3, 20 micron scale; Figures 4-9, 5 micron scale.

Figure 1. Early first-generation schizont with 3 nuclei showing relationship of host cell to reticular connective tissue cells surrounding crypt. A refractile body is present in cytoplasm of parasite; a crescent body in parasitophorous vacuole. From intestinal fistula fixed 3 days after introduction of sporozoites.

Figure 2. First-generation schizont of five-day infection showing flattened envelope of cells around portion of host cell and hypertrophied host cell, with cytoplasm arranged into 2 layers.

Figure 3. First-generation schizont of seven-day infection showing extension of parasitophorous vacuole and its fluid contents (heavy stippling) between infolding layers of nuclei during compartmentalization. Note host cell nucleus and envelope of flattened cells.

Figure 4. Host cell harboring first-generation trophozoite. A large nucleolus is present in the host cell nucleus. The parasite has a large refractile body (below) and a nucleus (above); a crescent body is present in parasitophorous vacuole. From three-day fistula.

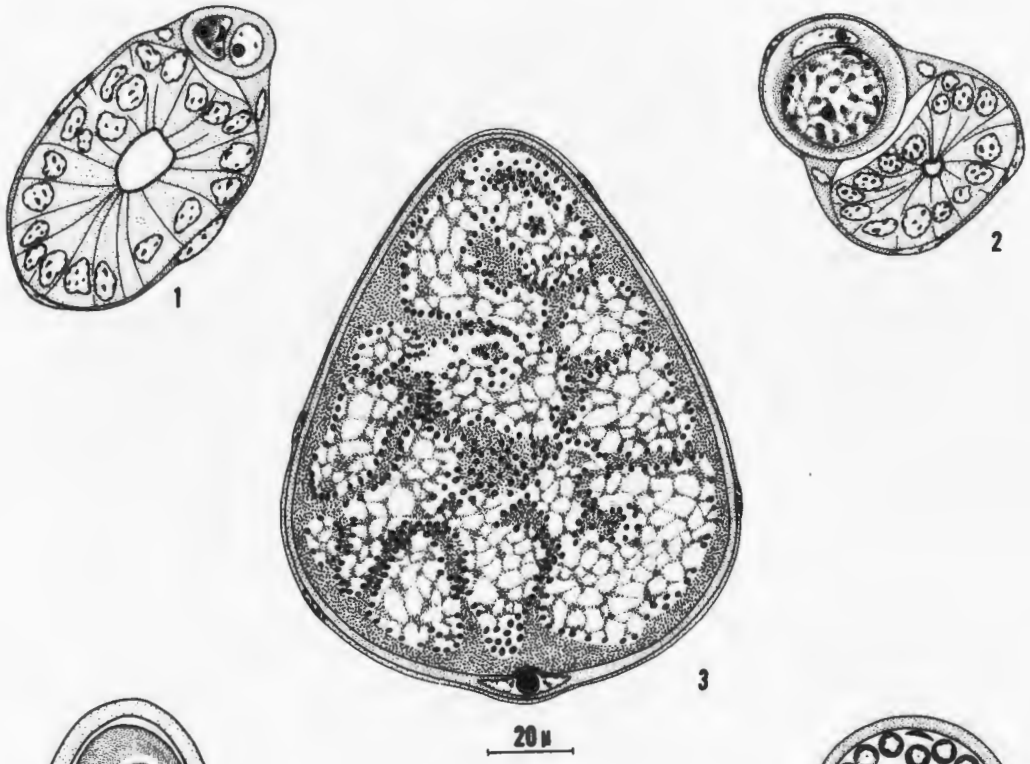
Figure 5. Macrophage in invaded schizont, with first-generation merozoites inside vacuole. Ileum, 12 days after inoculation.

Figure 6. Mature second-generation schizont in epithelial host cell showing some merozoites with crescent-shaped nuclei. A crescent body and residual body are present. Cecum, 11 days after inoculation.

Figure 7. Early trophozoites of second-generation schizont in epithelial host cell. A crescent body is present in parasitophorous vacuole. From 24-hr cecal biopsy.

Figure 8. Early macrogamete in epithelial host cell. A crescent body occurs in parasitophorous vacuole; a satellite body is located adjacent to parasite nucleolus. Colon, 12 days after inoculation.

Figure 9. Early microgametocyte in epithelial host cell. A crescent body occurs in parasitophorous vacuole. Colon, 13 days after inoculation.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Figures 10-42. Photomicrographs of endogenous stages of E. ninakohlyakimovae. Abbreviations: B, blastophore; C, crescent body; CR, cytoplasm of reticular connective tissue cell; EN, nucleus belonging to envelope of flattened cells; G, granule; HC, host cell cytoplasm; HN, host cell nucleus; HNU, host cell nucleolus; IC, inner layer of host cell cytoplasm; IN, indentation of crypt wall; L, accumulation of leucocytic cells; LC, lumen of crypt; M, merozoite; MB, forming merozoite; MG, microgametes; N, nucleus of parasite; NL, nucleus of leucocytic cell; NU, nucleolus of parasite; OC, outer layer of host cell cytoplasm; R, refractile body; RB, residual body; SB, satellite body; V, parasitophorous vacuole. Figures 10-23. Schizonts, stained with Feulgen method unless otherwise noted.

Figure 10. Schizont, from five-day infection, showing random arrangement of nuclei and characteristic indentation of crypt wall. X 640.

Figure 11. Same stage as in Figure 10, but showing stratification of host cell cytoplasm into two layers. Haematoxylin and eosin. X 640.

Figure 12. Schizont from six-day infection showing random arrangement of nuclei. Note large nucleolus (HNU) within hypertrophied host cell nucleus. X 640.

Figure 13. Same as in Figure 12, but showing portion of envelope of flattened cells surrounding host cell. Note nucleus of envelope cell. X 640.

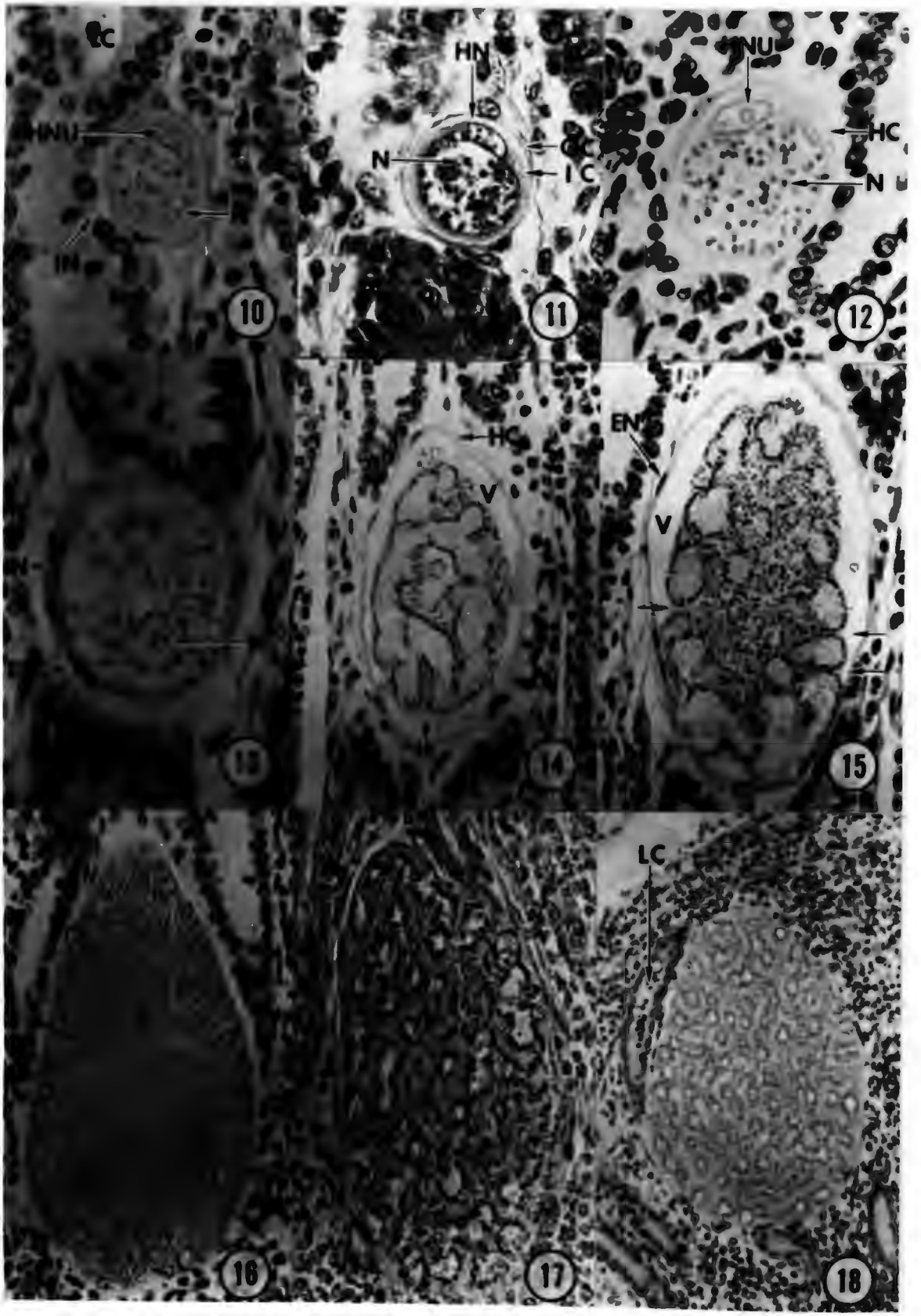
Figure 14. Schizont from seven-day infection, in early stage of compartmentalization. Note infolding of peripheral layer of nuclei (arrow). X 400.

Figure 15. Schizont as in Figure 14, but in more advanced stage of compartmentalization. Note infolding layers of nuclei (arrow). X 640.

Figure 16. Schizont from eight-day infection in late stage of blastophore formation. Small rings of nuclei represent blastophores (arrow). X 400.

Figure 17. Schizont as in Figure 16, but having rows of forming merozoites extending out from blastophores (arrow). Feulgen preparation, phase-contrast. X 400.

Figure 18. Nearly mature schizont from nine-day infection, with newly formed and/or incompletely formed merozoites which have not yet become randomly distributed. X 256.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Figures 19-23. Schizonts fixed in Zenker's and stained with Feulgen, unless otherwise noted.

Figure 19. Nearly mature schizont from nine-day infection; newly formed merozoites are becoming randomly distributed: this is seen especially in central area of schizont. X 256.

Figure 20. Mature schizont from ten-day infection; merozoites are randomly distributed throughout schizont. A moderate concentration of leucocytes surrounds the schizont. Haematoxylin and eosin, X 256.

Figure 21. Mature schizont in early stage of invasion by leucocytic cells; from ten-day infection, X 256.

Figure 22. Mature schizont in intermediate stage of invasion by leucocytic cells; from ten-day infection, X 256.

Figure 23. Mature schizont in advanced stage of invasion by leucocytic cells; from ten-day infection, X 256.

Figure 24. Site of destroyed schizont (arrow) in which macrophages and fibrocytic cells are present. From lamb killed 13 days after inoculation. Feulgen, X 256.


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Figures 25-33. Asexual stages of *E. [illegible]*, fixed in Zenker's and stained with haematoxylin and eosin unless otherwise noted.

Figure 25. Fresh, first-generation merozoite in extended position, from ten day infection. Phase-contrast, X 1600.

Figure 26. Fresh, first-generation merozoite in flexed position, from ten day infection. Phase-contrast, X 1600.

Figure 27. Trophozoite of first-generation schizont in fistula tissue, three days after inoculation, X 1600.

Figure 28. Young, first-generation schizont in fistula tissue showing attachment of adjacent reticular cells to host cell. Note characteristic indentation of crypt wall. Three days after inoculation, X 1600.

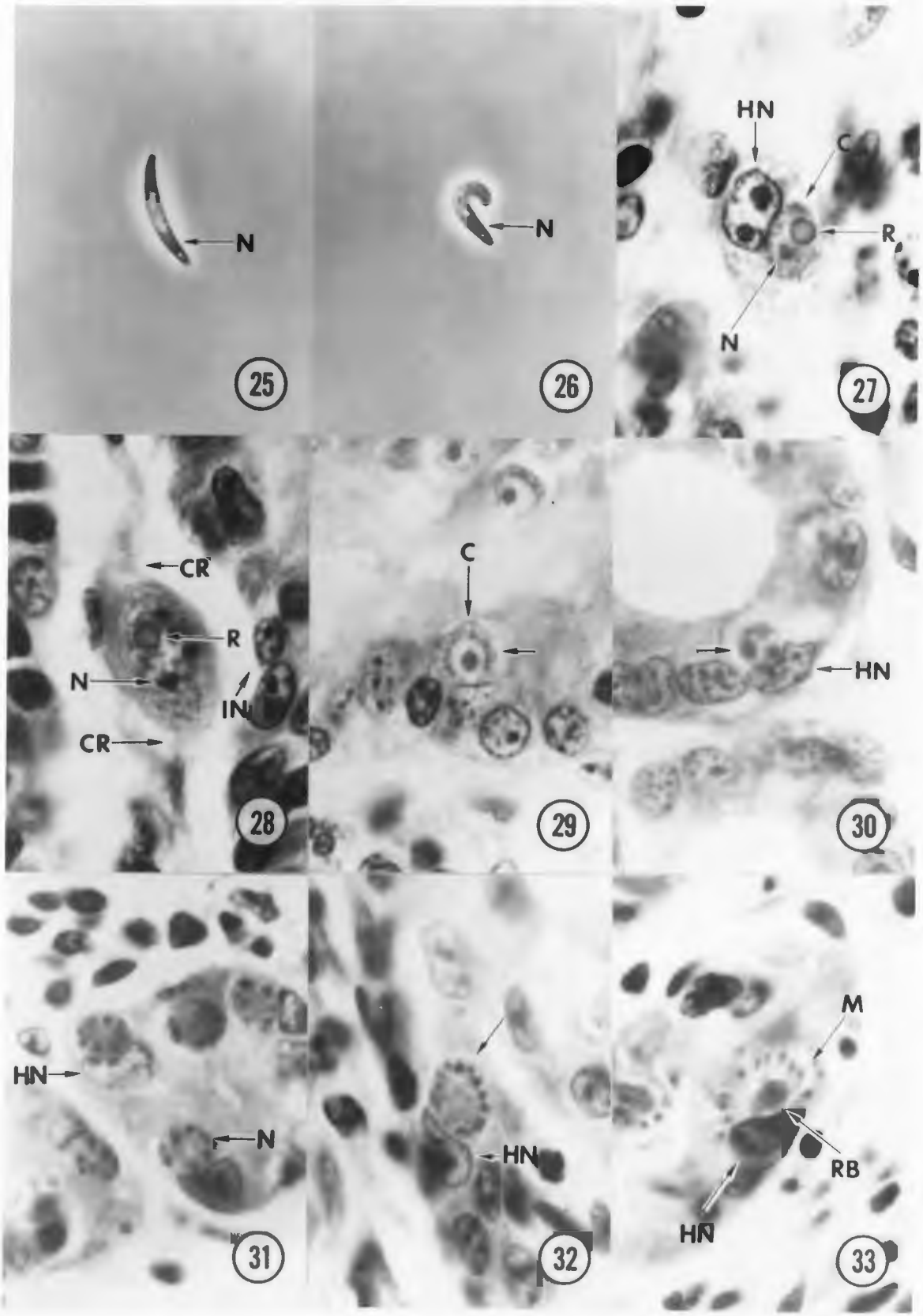
Figure 29. Trophozoite of second-generation schizont (arrow) in cecal biopsy tissue inoculated 24 hours earlier with first-generation merozoites, X 1600.

Figure 30. Binucleate second-generation schizont (arrow) in cecal biopsy tissue as in Figure 29. Note characteristic location of schizont in crypt epithelium. X 1600.

Figure 31. Intermediate second-generation schizont with the majority of nuclei in a peripheral location. 10 days after inoculation. Iron haematoxylin, X 1600.

Figure 32. Second-generation schizont in more advanced stage than that of Figure 31 and with all nuclei in a peripheral location. Note elevations at periphery. 10 days after inoculation. Iron-haematoxylin, X 1600.

Figure 33. Second-generation schizont with radially arranged immature merozoites still attached to eccentric residual body. 10 days after inoculation, X 1600.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Figures 34-42. Sexual stages in tissue sections from colon of lambs harboring 13-day infections unless otherwise noted. Fixed in Zenker's and stained with haematoxylin and eosin unless otherwise noted.

Figure 34. Uninucleate microgametocyte (arrow). X 1600.

Figure 35. Binucleate microgametocyte (arrow). X 1600.

Figure 36. Immature microgametocyte with peripheral arrangement of nuclei. X 1600.

Figure 37. Nearly mature microgametocyte with peripheral arrangement of nuclei. X 1600.

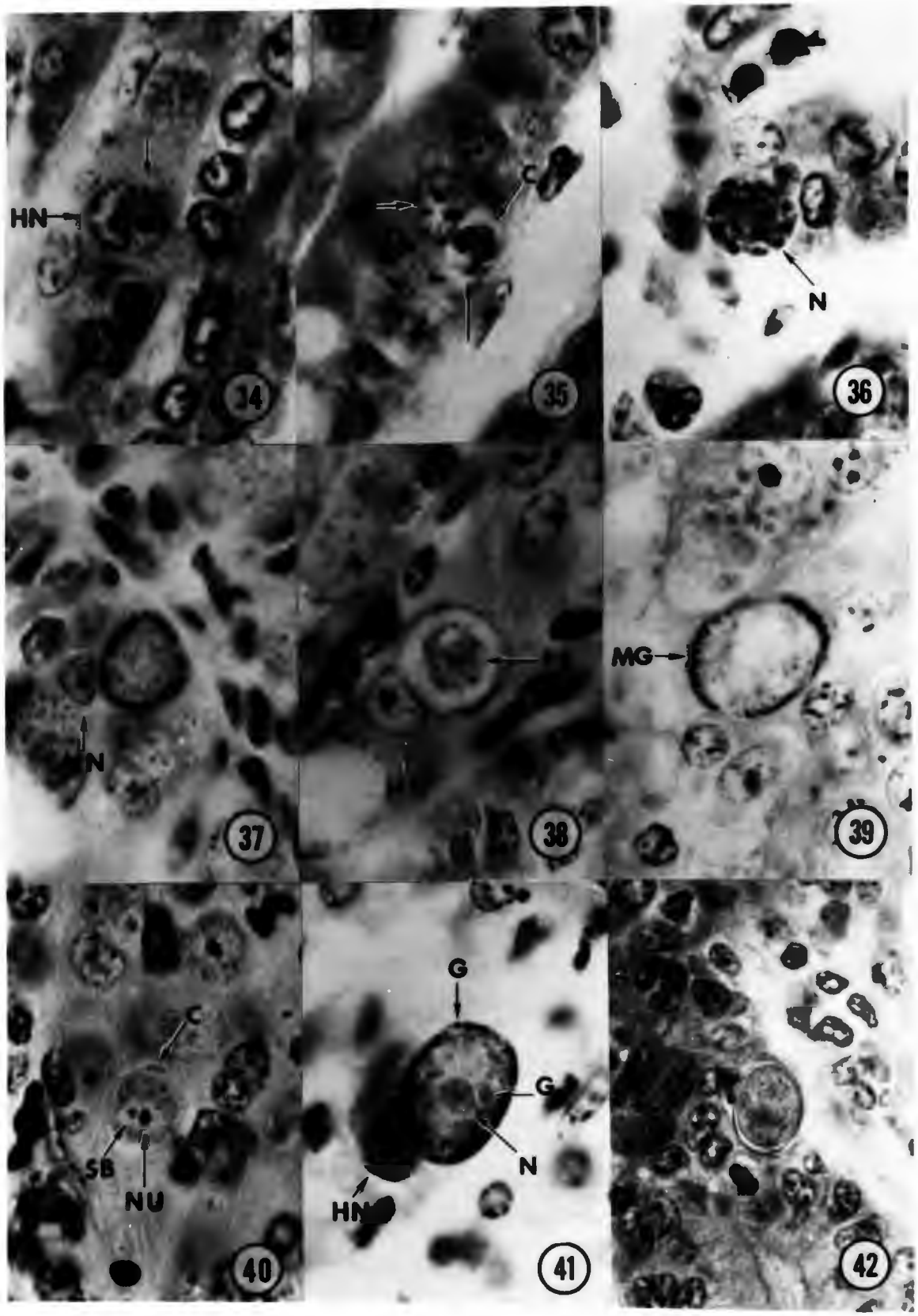
Figure 38. Mature microgametocyte with central residual body. Iron-haematoxylin, X 1600.

Figure 39. Mature microgametocyte with peripheral arrangement of microgametes (MG). X 1600.

Figure 40. Early macrogamete; note satellite body (SB) and nucleolus (NU). From 12-day infection. X 1600.

Figure 41. Mature macrogamete with lightly stained eosinophilic granules and darkly stained basophilic granules, at the periphery. X 1600.

Figure 42. Oocyst. X 1024.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DISCUSSION

Duration of Experimental Infection

The prepatent periods reported for Eimeria ninakohlyakimovae in sheep vary. In the present study a prepatent period of 11 days was observed in each of the 4 lambs studied. However, in other studies using lambs, prepatent periods of 10 days (Christensen, 1941), 10 to 11 days (Hammond et al. 1967), and 15 days (Shumard, 1957), have been reported. Balozet (1932) reported a prepatent period of 10 to 13 days for this species in goats. Sayin (1964) made no reference to the prepatent period for this species in goats.

Regarding the patent period for this species in lambs, in the present study no oocysts were observed in the feces on, or after, the nineteenth day post-inoculation in the two lambs studied. For 8 lambs however, Hammond et al. (1967) reported a considerably longer period of oocyst discharge, which terminated at about 26 to 28 days post-inoculation. Also, Shumard (1957) reported that in 3 lambs harboring mixed infections of E. ninakohlyakimovae and E. faurei, oocyst discharge in both species increased until the twenty-first day, and then gradually decreased.

Development of First-Generation Schizonts

The *in vivo* development of the large, first-generation schizont of *Eimeria ninakohlyakimovae* observed in sheep in the present study closely parallels that of the large first-generation schizont of *E. bovis* observed in cattle by Hammond et al. (1946), Hammond et al. (1963), and Hammond et al. (1966). This is illustrated in the following comparison.

Mature first-generation schizonts of both species attained an average size of about 300, had thousands of merozoites, and occurred in greatest concentration in the small intestine at about 10 feet anterior to the ileocecal valve. In both species a single crescent body and at least 1 refractile body were present in the early stages of schizogony. Also, an envelope of flattened cells of host origin surrounded the host cells of the schizonts relatively early in development. The reaction of the host cells to the developing schizonts was similar for both parasites, including an increase in the volume of the host cell cytoplasm and an appreciable increase in the size of the host cell nucleus and nucleolus. The chromatin material of the host cell nuclei became thinly distributed along the nuclear membrane, with a few small granules of chromatin scattered in the nucleoplasm, in contrast to the relatively large clumps in the nucleoplasm of uninfected cells. In both organisms the host cell cytoplasm became stratified

into two differentially staining layers. Further, the schizonts of these two organisms presented similar patterns of development with respect to merozoite formation. In each species infolding layers of nuclei formed progressively smaller compartments. These developed the blastophores from the periphery of which the merozoites were formed. Regarding the host response to the infections, a marked inflammatory reaction was present in the host tissues surrounding many of the mature first-generation schizonts of both species. Many of these schizonts were subsequently invaded and destroyed by the leucocytic cells associated with the inflammatory reaction. This reaction occurred earlier, more frequently, and possibly more rapidly in E. ninakohlyakimovae than in E. bovis. This difference might be associated with the deeper location in the mucosa of the former species. In E. bovis, the schizonts located near the base of the mucosa were invaded more frequently than those in the villus (Hammond et al. 1966). In both species, the leucocytic cells most commonly associated with invasion were eosinophils, neutrophils, and macrophages, and there appeared to be a disintegration of the envelope of flattened cells surrounding invaded schizonts. Clearly, the development of the first-generation schizonts of both species is basically similar. Nevertheless, some fundamental differences do exist. One of these differences concerns the type of host cell in which schizogony occurs. For E. bovis the host cell was

identified as an endothelial cell lining the central lacteal within the villus (Hammond et al., 1946), whereas for E. ninakohlyakimovae the host cell was identified as a reticular connective tissue cell in the lamina propria surrounding the base of the crypts of Lieberkuhn. However, it should be noted that both of these host cells are considered to be of mesodermal origin, thus the host cells are alike in that respect. No evidence was obtained in the present study that the host cell of large schizonts of E. ninakohlyakimovae was epithelial, as reported by Lotze (1954).

A second difference involves the time sequence in which first-generation schizogony occurred. In E. bovis infections, first-generation schizonts were seldom seen in vivo before 6 days post-inoculation, and mature schizonts were observed to occur at about 14 days post-inoculation (Hammond et al., 1946). The time interval between the appearance of young first-generation schizonts and the appearance of mature first-generation schizonts was about 8 days. This period was 6 days for E. ninakohlyakimovae, the first young schizonts being observed at about 4 days post-inoculation and the mature schizonts at about 10 days post-inoculation.

The relationship between the parasitophorous vacuole and the infolding layers of nuclei in the compartmentalization process appeared to represent a third major difference between the two species. Concerning this aspect of

development in E. bovis, Hammond et al. (1966) reported that a layer of clear cytoplasm surrounded the peripheral layer of nuclei within the schizont before and during compartmentalization, and that this clear cytoplasmic layer became invaginated between the infolding layers of nuclei. In E. ninakohlyakimovae, a layer of material, similar in appearance and location to the clear cytoplasmic layer reported by Hammond et al. (1966), was observed. However, this material as seen in E. ninakohlyakimovae was interpreted as being a vacuolar fluid contained within the parasitophorous vacuole. Thus, I believe that E. bovis resembles E. ninakohlyakimovae in this respect, so that the apparent difference does not exist.

The view that the parasitophorous vacuole extends between the infolding layers of nuclei suggests that the membrance which eventually encloses the blastophores and which becomes incorporated as the outer membrane of the newly formed merozoites (Sheffield and Hammond, 1966) is an extension of the limiting membrane of the trophozoite and therefore derived from it. In this respect, the basic pattern of schizogony in E. ninakohlyakimovae and E. bovis would appear to be consistent with that observed in Plasmodium species by Aikawa, Huff, and Sprinz (1967), Hepler, Huff, and Sprinz (1966), and Vickerman and Cox (1967).

Other species of Eimeria having large schizonts of 150 or more in diameter have been reported from sheep. They are E. ahsata, E. arloingi, E. gilruthi, and E. parva

(Davis, Downan, and Smith, 1963; Levine, 1961; Pellérdy, 1965). In three of these species certain authors have given some information as to the in vivo pattern of schizogony, which appeared to be similar to that seen in E. ninakohlyakimovae. For E. arloingi, Lotze (1953) stated that at a certain stage of development, the nuclei of the large schizonts were arranged in rows describing various configurations. His observations suggest that compartmentalization occurred in the schizonts of E. arloingi. Kotlán et al. (1951) reported the formation of nests and spheres of nuclei in the large schizonts of E. parva, an observation which indicates the formation of blastophores in the species. Chatton (1910) and Triffit (1925) both stated that nuclei of E. gilruthi schizonts became arranged in mulberry-form groups which then formed into spheres, called blastophores by Chatton. From these spheres merozoites grew radially. These events reported for E. gilruthi strongly suggest a pattern of schizogony similar to that reported for E. ninakohlyakimovae in the present study.

Apparently, however, not all large schizonts of Eimeria develop according to this pattern. In cattle, Chobotar et al. (1969), studying the in vivo process of schizogony in the large first-generation schizont of E. auburnensis, found a slightly different pattern of schizogony. They found that a layer of peripherally distributed nuclei invaginated in association with the parasitophorous vacuole

at various points along the layer, but that these invaginations did not progress to the extent that either compartmentalization or blastophore formation occurred. The invaginations of the peripheral layer of nuclei in E. auburnensis resembled rather large cylindrical tubes growing into the central areas of the schizont. Merozoites budded into the parasitophorous vacuole which formed the central cavity of each tube; each merozoite then incorporated the nucleus associated with it.

Concerning the invasion of mature first-generation schizonts by leucocytic cells, Hammond et al. (1966) stated that the invasion process ended in the destruction of the schizont and its merozoites. The results of the present study indicate that in E. ninakohlyakimovae the schizonts are destroyed by invading leucocytic cells, and that a phagocytosis of merozoites by macrophages is a primary mechanism by which that destruction is accomplished. The microscopic appearance of a field of macrophages with recently phagocytized merozoites is one of small individual clusters of merozoites. Except for its nucleus, the cell outline of the macrophage is relatively inconspicuous. Although Hammond et al. (1966) made no mention of this phagocytic process in E. bovis, their reference to the isolation of merozoites into small groups suggests that they probably did observe it. In view of the similarity of the invasion process in both species there is little reason to suspect that phagocytosis of merozoites by macrophages does

not occur in invaded schizonts of E. bovis.

The role of the macrophage-sporozoite association which exists in Eimeria species of chickens has been studied by several authors, viz., Van Doorninck and Becker (1957), Cudley and Burns (1959), Patillo (1959), and Doran (1966).

There is general agreement among these authors that macrophages which harbor sporozoites may transport these sporozoites to the definitive sites in the host tissue of the chicken. The sporozoites then, presumably, become freed from the macrophages and develop into schizonts. Nothing was seen in E. ninakohlyakimovae in the present study which indicated that macrophages with merozoites functioned in this capacity. To assign any specific function to the macrophage-merozoite relationship in E. ninakohlyakimovae, other than destruction of the merozoites, at this time would undoubtedly be premature, but this relationship may also play a role in the immune process.

First-Generation Merozoites

Light microscope observations of the first-generation merozoites of E. ninakohlyakimovae were similar to those of E. bovis as reported by Hammond et al. (1965). No distinct differences were observed in the distribution of chromatin within the nucleus or in the distribution of PAS-positive and RNA-positive granules in the cytoplasm.

The flexing and gliding movements were similar in the two species. The salient differences between the merozoites of these species were those of size and shape. In general, the merozoites of E. ninakohlyakimovae appeared slightly shorter and blunter than those of E. bovis. On the basis of measurements of living specimens, merozoites of E. ninakohlyakimovae were 1.6 shorter and 0.7 wider than those of E. bovis; merozoites of E. ninakohlyakimovae were only 0.3 shorter than those of E. auburnensis (Chobotar et al., 1969).

Development of Second-Generation Schizonts

The development of second-generation schizonts of E. ninakohlyakimovae was similar to that of E. bovis as reported by Hammond et al. (1963). In both species the schizonts develop in the epithelial cells of the crypts of the cecum and colon. Also schizonts of both species have a relatively rapid development, with the time necessary to produce mature schizonts being only 1 to 2 days. Some important differences between the second-generation schizonts of the two species include the number and size of the merozoites. Mature second-generation schizonts of E. bovis averaged 10 in maximum diameter and had 30 to 36 merozoites, with an average length of 3.5. Those of E. ninakohlyakimovae averaged 12 in maximum diameter and had 22 to 30 merozoites with an average length of 5.5.

Additional differences include the presence of a

crescent body in the second-generation schizonts of E. ninakohlyakimovae and the crescent-shaped appearance of the chromatin in the nuclei of second-generation merozoites. Neither of these features have been reported for E. bovis. However, crescent-shaped nuclei were observed in the schizogonous stages of E. nieschulzi in rats by Matsubayashi (1938), and in E. intestinalis in rabbits by Cheissin (1958). The significance of this crescent-shaped chromatin in the nuclei is unknown. In calves harboring 12- and 14-day infections with E. auburnensis, Chobotar and Hammond (1969) observed second-generation schizonts in the lamina propria of the small intestine. Mature second-generation schizonts of E. auburnensis averaged 8.5 in maximum diameter and had 4 to 11 merozoites with an average length of 7.9. Neither a crescent body nor a residual body was reported to occur in the second-generation schizont of E. auburnensis. Thus, E. bovis, E. auburnensis, and E. ninakohlyakimovae are similar in that the life cycles include a large first-generation schizont and a small second-generation schizont. As compared with first-generation schizogony, that of the second-generation appears relatively uncomplicated in that no compartmentalization occurs. However, the formation of the second-generation merozoites in E. ninakohlyakimovae appears to be similar to that reported for the formation of first-generation merozoites from individual blastophores in E. bovis by Hammond et al. (1966).

Some authors have found small schizonts existing concurrently with E. ninakohlyakimovae infections. Therefore, the possibility existed that these small schizonts may have been part of the life cycle of that species. In goats harboring infections with E. ninakohlyakimovae, Baloset (1932) reported the presence of small schizonts 15 to 35 in diameter, which had 40 to 200 merozoites. These were observed late in the infection and were associated with the gametocytes. Sayin (1964), also working with goats harboring infections with E. ninakohlyakimovae, observed small schizonts 32 to 43 in diameter. Sayin made no reference to the number of merozoites they had, but he did say that the merozoites measured 11 by 1. The schizonts reported by Baloset and Sayin were larger than the second-generation schizonts reported in the present study. Also, the number and size of merozoites reported by Baloset were in excess of those of the second-generation schizont of the present study. Therefore, it is doubtful that the schizonts which they observed in goats belonged to E. ninakohlyakimovae unless development of this species occurs differently in different hosts. Levine, Ivens and Fritz (1962) also observed small schizonts in epithelial cells of the crypts of Leiberkuhn in the jejunum of a month-old goat harboring infections with E. arloingi, E. crandalis, and E. christenseni. The schizonts averaged 12 in maximum diameter and had 16 to 22 merozoites with an average length of 9. Because of the location of the schizonts, the large

size of the merozoites, and the goat's apparent lack of infection with E. ninakohlyakimovae, it is unlikely that the small schizonts observed by Levine et al. (1962) belonged to E. ninakohlyakimovae.

Development of Gametocytes

Hammond et al. (1946) found that the sexual stages of Eimeria bovis occurred in the epithelial cells lining the crypts of Lieberkuhn in the cecum and large intestine of cattle. The earliest sexual stages of E. bovis were observed in the tissues at 17 days post-inoculation, and oocyst discharge began at 18 days post-inoculation. In E. ninakohlyakimovae in the present study, sexual stages were observed to undergo development in comparable tissues, but the earliest gametocyte stages were observed at about 11 days post-inoculation and oocyst discharge began on day 12. Microgametogenesis in E. ninakohlyakimovae was similar to that observed in E. bovis. The nuclei of the microgametocytes of both species assumed a peripheral distribution, and having done so, the chromatin of each nucleus became crescent-like in appearance, underwent elongation, and was then incorporated into the developing microgamete. A similar arrangement of chromatin was observed in the formation of microgametes of E. caviae by Lapage (1940) and of E. auburnensis by Chobotar and Hammond (1969). However, the nuclei of the microgametocytes of E. auburnensis became arranged at the periphery of compartments; this was

associated with the large size of the microgametocyte.

A large, prominent nucleolus and a satellite body were present in the nucleoplasm of young macrogametes of E. ninakohlyakimovae. These structures were also observed in the nucleoplasm of macrogametes of E. bovis by Hammond et al. (1946), of E. auburnensis by Chobotar and Hammond (1969), and of E. magna by Cheissin (1960). The significance of the satellite body has not been determined, although Cheissin (1960) found that it stained blue with bromophenol blue in E. magna. The observation that the cytoplasmic granules which occur in the macrogametes of Eimeria species play a part in oocyst wall formation has been discussed by several authors, viz., Scholtyseck and Voigt (1964), Löser and Gönnert (1965), Scholtyseck, Hammond and Ernst (1966), and Scholtyseck, Rommel, and Heller (1969). In macrogametes in the present study, cytoplasmic granules were observed which stained eosinophilic, whereas others were basophilic with haematoxylin and eosin stain. These granules appeared to participate in oocyst wall formation. In E. auburnensis, Chobotar and Hammond (1969) found that the oocyst wall was apparently formed chiefly by the fusion of eosinophilic plastic granules, with probable participation of basophilic granules in formation of wall membranes.

A single crescent body was present in the parasitophorous vacuoles of microgametocytes and macrogametocytes of E. ninakohlyakimovae. Crescent bodies have not been

reported in the gametocytes of E. bovis; however, Chobotar and Hammond (1969) did observe their presence in the gametocytes of E. auburnensis.

As indicated in the preceding discussion the numerous similarities between the endogenous stages of E. ninakohlyakimovae and E. bovis are striking. There is little reason to doubt the existence of a close phylogenetic relationship between these two species. The exact nature and degree of this relationship, however, needs further study.

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